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Elevated Serum Neutrophil Gelatinase-Associated Lipocalin Is an Early Predictor of Severity and Outcome in Acute Pancreatitis

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Abstract

OBJECTIVES—About 210,000 new cases of acute pancreatitis (AP) involving reversible inflammation of the pancreas are reported in the United States every year. About one-fourth of all patients with AP go on to develop severe acute pancreatitis (SAP), which, unlike uncomplicated or mild acute pancreatitis (MAP, usually a self-limiting disease), constitutes a life-threatening condition with systemic complications, chiefly multiorgan dysfunction. An early prediction of the severity and outcome of patients with acute pancreatitis (AP) can lead to better treatment regimens for patients with SAP. There is currently no established biomarker for the early diagnosis of SAP. In this study, we investigated the potential of serum neutrophil gelatinase-associated lipocalin (NGAL) as an early marker to distinguish severe (SAP) from MAP and examine its ability to predict the prognosis of patients with SAP.

METHODS—To check the time kinetics of rise in NGAL during AP, we quantified NGAL levels in sera from mice with MAP or SAP at various time points (6, 12, 24 and 48 h) using sandwich enzyme-linked immunosorbent assay. NGAL levels were also quantified in serum from 28 MAP and 16 SAP cases and compared with 28 chronic pancreatitis and 30 healthy control samples. Samples collected within 5 days from onset of symptoms were included. The relationship of NGAL levels with survival and multiorgan failure (MOF) in SAP was also examined.

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Specific author contributions: Subhankar Chakraborty, Sukhwinder Kaur, and Neil Sharma performed the NGAL ELISAs on the human and mouse serum samples. Subhankar Chakraborty performed the statistical analysis and wrote the paper. Venkata Muddana, Georgios I. Papachristou, Randall E. Brand, and David Whitcomb were involved in recruiting the patients and coordinated the collection of samples and assembling the patient information. Uwe A. Wittel performed the animal procedures and collected the serum samples from the mice. Surinder K. Batra helped to design and coordinate the study. All authors were involved in reviewing the results and the paper.

CONFLICT OF INTEREST **Guarantor of the article:** Surinder K. Batra, PhD.

Potential competing interests: None.

RESULTS—Although NGAL levels were significantly higher in mice with both MAP and SAP 6 h after induction (compared to control animals), only mice with SAP exhibited a significant increase in NGAL levels at 24 h ($P=0.003$). NGAL levels declined at 48 h after induction in animals with both MAP and SAP but did not reach baseline levels. Among patients, mean ($\pm$ s.e.) serum NGAL level was significantly higher in SAP (634 ± 139 ng/ml) compared to MAP (84.7 ± 7 ng/ml, $P=0.0001$). On subanalysis, the difference between MAP and SAP cases was significant in the first 48 h but not at 72, 96, or 120 h. NGAL was 100%, 96%, 97%, and 84% specific and 100%, 87.5%, 92%, and 94% sensitive in distinguishing SAP from MAP at 48, 72, 96, and 120 h, respectively, after the onset of symptoms. NGAL levels were significantly higher in SAP cases complicated by MOF ($P=0.004$), and high NGAL levels in SAP appeared to correlate with a fatal outcome.

CONCLUSIONS—Our data provide the first evidence for the potential of serum NGAL as an early marker to distinguish MAP from SAP. Further, high NGAL levels predict MOF and fatal outcome in patients with SAP. This study provides sufficient evidence for multi-institutional randomized trials for estimating the potential of NGAL as early biomarker for SAP.

INTRODUCTION

Acute pancreatitis (AP) is a reversible inflammation of the pancreas that is either localized to the pancreas or may spread to involve adjoining tissues and remote organs (1). An estimated 210,000 new cases of AP are reported in the United States every year (2). About one-fourth of all patients with AP go on to develop severe acute pancreatitis (SAP), which unlike uncomplicated or mild acute pancreatitis (MAP, usually a self-limiting disease) constitutes a life-threatening condition with systemic complications, chiefly multiorgan dysfunction (3,4). The management of AP is still challenging chiefly owing to the delay in hospitalizing patients after the onset of symptoms and the difficulty in discriminating MAP from SAP, especially in the first 48–72 h (3). Several clinical multifactorial scoring systems, including the Ranson's, Glasgow, and Acute Physiology and Chronic Health Evaluation-II (APACHE-II) scores, are currently used to predict the severity and clinical outcome of these patients with an accuracy of up to 80% (1,5,6). In addition, several biochemical markers have also been examined for their ability to predict the severity of AP including C-reactive protein (CRP), procalcitonin, trypsinogen activation peptide, and interleukin-6 (IL-6) (reviewed in (7,8)). However, their utility in predicting an outcome or triaging patients for admission to the intensive care units remains limited (9). CRP, the most commonly used biochemical marker to distinguish SAP from MAP is elevated only 3–4 days after the first onset of symptoms (5). Thus, there is an urgent need for novel surrogate markers that can identify patients with SAP, preferably within 48 h of onset of symptoms.

Neutrophil gelatinase-associated lipocalin (NGAL; also known as human neutrophil lipocalin, lipocalin-2, siderocalin, 24p3, or LCN2) is a 24 kDa glycoprotein belonging to the lipocalin superfamily of secreted and cytosolic proteins that carry hydrophobic ligands (6). It is released from activated neutrophils at the sites of infection and inflammation where it functions by sequestering bacterial iron binding proteins called siderophores. By this mechanism, NGAL behaves as a bacteriostatic agent in acute infections. NGAL is also expressed by normal epithelial cells in a number of tissues, including the small intestine, stomach, colon and kidney. However, it is not expressed by the normal pancreatic ducts or acini (10). In clinical practice, NGAL is a widely accepted early marker of acute renal injury (11).

We have previously reported that NGAL is expressed *de novo* during the progression of pancreatic adenocarcinoma. We also observed in the same study that NGAL levels were significantly elevated in the sera of patients with pancreatitis and pancreatic cancer compared to matched healthy controls (12). Here, we report that quantitative measurement of NGAL in serum samples is highly sensitive and specific in distinguishing patients with MAP from SAP.

Interestingly, NGAL is also a very early marker of severity in AP, being elevated within 6 h of induction of SAP in mice model. Although several studies have previously investigated the time course of biomarkers in body fluids (chiefly serum or plasma and urine) of patients with AP, we report the time course of a biomarker in the animal model that recapitulates closely the pathophysiology of SAP and validate its diagnostic and prognostic significance in patient samples in the same study.

METHODS

Study population

The severity in AP study protocol was approved by the Institutional Review Board (IRB) of the University of Pittsburgh Medical Center (IRBs 209-00-EP and EH 02-185). Informed consent was obtained from all patients and controls before enrollment into the study. The diagnosis of AP was based on two of the following three features: (i) abdominal pain characteristic of AP, (ii) serum amylase and/or lipase ≥ 3 times the upper limit of normal, and (iii) characteristic findings of AP on a CT scan. Patients were recruited within 24 h from the time of admission. Patients were classified into two groups, MAP and SAP, based on the presence of organ failure for more than 48 h in the latter. Organ failure was defined as the presence of any one of the following three criteria: shock (systolic blood pressure < 90 mm Hg), pulmonary insufficiency (arterial $PO_2 < 60$ mm Hg at room air or mechanical ventilation), or renal failure (serum creatinine level ≥ 2 mg/dl after rehydration or on hemodialysis). Patients with organ failure affecting more than one of the three systems were classified as having multiorgan failure (MOF).

Study design

The 44 patients with AP included in this pilot study had blood samples obtained at admission and then on a daily basis. Of the 44 patients, 11 (25%) had recurrent pancreatitis, whereas in 33 patients (75%) this was the initial episode of AP. Serum samples were stored at -80°C . Serum CRP was measured using the Luminex extracellular assay (Invitrogen, Carlsbad, CA). APACHE-II scores were recorded on admission and at days 1, 2, 3, 4, and 7 after admission. Ranson's score was recorded 48 h after admission. After the end of the study, each patient's data were reviewed to ascertain eligibility for inclusion into the study. Serum samples collected more than 5 days from the time of onset of symptoms were excluded from the study.

Mouse model of SAP

All animal procedures were conducted according to the Federation of European Laboratory Animal Science Associations guidelines and approved by the local animal welfare committee. A mouse model that closely mimics the pathophysiology of SAP seen in humans was described by our group earlier (13). In this model, infusion of the common bile duct (CBD) with sodium taurocholate induces a severe form of pancreatitis accompanied by MOF resembling the clinical situation seen in patients with SAP. Microscopically, pancreatic acinar damage, including acinar cell necrosis, hemorrhage, and leukocyte infiltration, chiefly involving the head of the pancreas, is observed. These histological changes reach a peak 48 h after the induction of pancreatitis. Conversely, saline infusion into the CBD initially (i.e., in the first 24 h) induces extensive pancreatic edema without necrosis or hemorrhage, followed later by a low-grade leukocyte infiltration (mild edematous pancreatitis). Animals injected with intraperitoneal saline (hereafter referred to as control animals for the purpose of this study) exhibit no histological or gross evidence of pancreatic injury. This model was used in this study to investigate the potential of NGAL as an early marker (within the first 24 h) of SAP. Serum samples were immediately frozen at -80°C until further processing. The pancreatic histology of paraffin-embedded tissue was graded according to the score previously published by Spormann (14).

Determination of serum NGAL levels by sandwich ELISA

NGAL levels in serum were measured quantitatively by sandwich enzyme-linked immunosorbent assay (ELISA) using the NGAL Rapid ELISA Kit for human NGAL (AntibodyShop, Gentofte, Denmark) or the DuoSet ELISA kit for mouse NGAL (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. ELISA plates were read at 450 nm, and data collected were analyzed using the SOFTMAX PRO software (Molecular Devices, Sunnyvale, CA).

Statistical analysis

Data were analyzed using the MedCalc for Windows version 9.6.4.0 software (MedCalc Software, Mariakerke, Belgium). Age, body mass index (BMI), serum levels of NGAL, CRP, APACHE-II score, and Ranson's score were analyzed as continuous variables, whereas other variables were considered to be dichotomous. Continuous variables are presented as the mean with 95% confidence intervals (CIs). The fixed terms analyzed were severity and time (24, 48, 72, 96, and 120 h). Time points were measured from the first onset of symptoms to the time of collection of the serum. Receiver-operating characteristic curves (ROC curves) were constructed to determine the optimum cutoff for NGAL levels that could discriminate MAP from SAP. The area under the curve (AUC) was used as a measure of the ability of the test to distinguish between the two groups (15). Categorical variables were compared using the Fisher's exact test. Continuous variables were compared using the two-tailed Student's *t*-test, or the Kruskal–Wallis analysis of variance. The associations between variables were assessed by Spearman rank order correlation analysis. We considered a *P* value ≤ 0.05 as statistically significant.

RESULTS

Increased secretion of NGAL in a mouse model of SAP

To investigate the time course of NGAL levels in serum during an episode of AP, we injected mice with either intraperitoneal saline (control) or infusion of saline (MAP) or taurocholate (SAP) into the CBD. The Spormann score, which is an indicator of pancreatic inflammation and acinar cell damage, was significantly greater in mice with SAP ranging from 13.0 to 17.8 compared to 0.7 to 4.1 in mild and 0 to 0.3 in control animals ($P < 0.001$, representative histology from the three groups of animals in Figure 1, bottom). At 6h after the procedure, the levels of NGAL were significantly higher in the sera of mice with AP (both mild and severe) compared to that in the control animals (Figure 1, top). Serum NGAL levels in mice treated with SAP were significantly higher than those with MAP at 24 ($P=0.003$) and 48 h ($P=0.03$), but not at 6 or 12 h after the procedure.

In the mice with SAP, serum NGAL levels showed a significant increase with time, reaching peak levels 24 h after the treatment ($1,670 \pm 20$ ng/ml at 24h compared to 436 ± 52 ng/ml at 6h, $P=0.003$). Thereafter, the NGAL levels began declining, although the mean serum level at 48 h was still significantly higher than that in control animals at the same time point ($1,497 \pm 37$ ng/ml in the SAP group compared to 25 ± 17 ng/ml in control group, $P=0.005$).

In the mice with MAP, however, there was no significant variation in NGAL levels at 24 h compared to the initial sample (700 ± 61 ng/ml at 24h vs. 724 ± 22 ng/ml at 6h, $P=0.94$). Notably, the NGAL level showed a significant decline at 48 h compared to that at 24h (158 ± 17 ng/ml at 48h, $P=0.02$). The levels of NGAL in the three groups of mice at different time points are summarized in Table 1.

Elevated level of NGAL in patients with AP

To validate the findings from the mouse model, we analyzed NGAL levels in a group of 102 subjects comprising 44 patients with AP, 28 with chronic pancreatitis (CP), and 30 healthy individuals in a single-center pilot study. Of the 44 patients with AP, 28 were determined to have MAP and 16 to have SAP according to the criteria outlined in methodology. Of the 16 patients with SAP, 5 had samples drawn at more than one time point, whereas for the remaining 11, samples were obtained only at one time point. Ten patients diagnosed with MAP also had samples obtained at more than one time point.

The characteristics of the patients with AP included in the study are summarized in Table 2. The mean age in the patients with CP and healthy subjects was 48 ± 3.9 and 48.1 ± 2.1 years, respectively. Forty-three percent of the CP cases and 37% of healthy controls were men. Ninety-three percent of both CP and healthy controls were whites. Mean ($\pm$ s.e.) BMI in AP, CP, and healthy controls was 30.2 ± 1.05 , 25.2 ± 1.15 , and 29.5 ± 1.2 , respectively. There was no significant difference in BMI between patients with MAP and SAP.

The mean ($\pm$ s.e.) serum level of NGAL in the patients with SAP was 634 ± 139 ng/ml (range, 97–1,366 ng/ml; 95% CI, 388–865 ng/ml) that was significantly higher than that in patients with MAP (111 ± 13 ng/ml, $P=0.0001$), CP (122.2 ± 45 , $P<0.0001$), and the healthy controls (51 ± 5 ng/ml, $P=0.0001$). When grouped according to the time of collection of the sample, the mean ($\pm$ s.e.) serum NGAL level in patients with SAP was highest within the first 48 h after the onset of symptoms ($1,314 \pm 151$ ng/ml). This was significantly higher than the NGAL levels at 72, 96, or 120 h. However, no such trend was observed in the patients with MAP. Further, the mean NGAL level in patients with SAP was significantly higher than that in MAP cases in the first 48 h after onset of symptoms ($P=0.003$), but not later (72, 96, or 120 h) (Table 3).

There was no difference in NGAL levels in patients with AP (either SAP or MAP) when patients were grouped according to sex, race, or history of ethanol consumption. Further, no significant correlation was observed between mean NGAL levels and either age or BMI. The mean APACHE-II score at admission, the peak APACHE-II score, Ranson's score at 48 h, and serum CRP levels were observed to be significantly higher in SAP compared to that in MAP cases ($P<0.05$, Table 2). The level of serum amylase and lipase did not significantly differ between the two groups.

The optimum cutoff of serum NGAL level that distinguished SAP from MAP cases (grouped according to the time of collection of the samples) was analyzed by constructing a receiver-operating characteristic curve. Serum samples were grouped into those collected within 48, 72, 96, or 120 h from the onset of symptoms. The AUC for NGAL was observed to be 1.00 (95% CI, 0.81–1.00) at ≤ 48 h, 0.892 (95% CI, 0.74–0.97) at ≤ 72 h, 0.926 (95% CI, 0.81–0.98) at ≤ 96 h, and 0.929 (95% CI, 0.81–0.98) at ≤ 120 h, respectively (Table 4). The optimum cutoffs that distinguished SAP from MAP at these time points were 175.3, 125.6, 125.6, and 113.6 ng/ml (sensitivity and specificity of 100% and 100%; 87.5% and 96.4%; 91.7% and 97%; and 93.8% and 84.2%, respectively).

NGAL has been shown to be an early marker of acute renal injury. To examine whether the elevation of serum NGAL level in patients with SAP was a reflection of an underlying renal damage, we compared serum levels between patients with serum creatinine above or below a cutoff of 1.5 mg/dl (based on the range of baseline creatinine levels in normal individuals suggested by the Second International Consensus Conference of the Acute Dialysis Quality Initiative Group) (16). Serum creatinine was estimated in the same samples used to analyze NGAL levels. There was no significant difference in the mean NGAL levels between patients with serum creatinine > 1.5 mg/dl (744 ± 187 ng/ml) compared to those with serum creatinine ≤ 1.5 mg/dl (425 ± 197 ng/ml, $P=0.26$).

Seven patients with SAP subsequently developed single organ failure and nine developed MOF; of the latter, three patients died. There was no mortality in the cohort of patients with MAP. To determine the function of NGAL as a prognostic marker in SAP, we examined serum NGAL levels in patients with SAP who developed MOF compared to those who did not. Only patients with SAP who had at least one sample drawn within the 5 days of onset of symptoms were considered. For patients who had samples drawn at more than one time point, the sample drawn at the earliest time point was considered. Eleven patients with SAP met these criteria, five of whom developed MOF and six did not. Serum NGAL levels were significantly higher in patients with SAP with MOF (906 ± 195 ng/ml) compared to those without MOF (179 ± 42 ng/ml, $P=0.004$). We also compared other patient-associated factors (age, BMI, and length of stay in the hospital), clinical scoring systems (Ranson's score at 48 h, APACHE-II score at admission, and the peak APACHE-II score), and biochemical parameters (serum amylase, lipase, CRP, and serum creatinine at the time of admission and peak serum creatinine) between these two groups of patients (Table 5). The peak APACHE-II score ($P=0.008$) and the peak serum creatinine ($P=0.024$) were the only two variables significantly higher in patients with MOF. There was no significant correlation between the occurrence of MOF and either the age or race of the patient. Due to the small sample size, a regression analysis could not be performed.

In our study, three patients with SAP died during the course of their disease. These patients (designated as cases 1, 2, and 3) had their first serum samples drawn at 5, 3, and 2 days, respectively, from the time of onset of symptoms. We compared multiple prognostic variables in these three nonsurvivors with other patients with SAP who survived (designated "survivors") and had serum samples drawn at the same time point. Cases 1 and 2 were older (80 vs. 59.3 ± 7.2 years and 69 vs. 39 ± 6 years, respectively) and had a higher BMI (32.5 vs. 29.2 ± 2.4 and 30.2 vs. 29.5 ± 1.5 , respectively) than the surviving counterparts. Case 3, however, was similarly aged (53 vs. 52.7 ± 13.6 years) but had a higher BMI (32.1 vs. 27.7 ± 5.5) compared to the three surviving counterparts. Serum NGAL levels were 9.2-, 5.3-, and 1.5-fold higher in cases 1, 2, and 3 than the mean NGAL levels in the survivors. The serum creatinine was also elevated in all the three nonsurvivors, being 2.3-, 3.4-, and 3.5-fold higher in case 1, 2, and 3, respectively, than the mean serum creatinine in the corresponding survivors. The Ranson's score, peak APACHE-II score, and plasma CRP levels, however, did not show a consistent variation between the nonsurvivors and survivors (Figure 2a–c).

DISCUSSION

SAP is responsible for the morbidity and mortality caused by AP. Current management strategies for patients with SAP include early admission to intensive care units, vigorous intravenous resuscitation, urgent endoscopic retrograde cholangiopancreatography when cholangitis or biliary obstruction is present, antibiotic prophylaxis in patients with pancreatic necrosis, and close patient monitoring (3,7). Morbidity and mortality in SAP is divided in two phases: the early phase, characterized by the systemic inflammatory response syndrome, which can lead to MOF within the first week, and the late phase, characterized by infectious complications of pancreatic necrosis, which generally develops after the first 2 weeks of the disease (3). There have been numerous efforts in recent years to identify objective markers that can predict the severity of AP at the time of hospital admission. However, except for CRP, their utility in predicting the outcome or triaging patients for admission to the intensive care unit remains limited (9). A new prognostic scoring system, the bedside index for severity in AP (BISAP) has recently been introduced as an alternative to the existing APACHE-II, Ranson's, and computed tomography severity index scoring methods. A recent study comparing the ability of these four scoring systems to predict severity, pancreatic necrosis, and mortality among patients with AP found that the BISAP score was simpler and as accurate (AUC for predicting SAP was 0.81 for BISAP, 0.78 for APACHE-II, 0.84 for computed tomography severity index, and 0.94 for Ranson's score) as the other three scoring systems in predicting

patient prognosis (17). There is thus an urgent need for an early and accurate prediction of the severity of AP to ensure timely interventions in a specialized care setting. Here, we tested the hypothesis that serum NGAL levels are elevated early during the course of AP and correlate with the severity of the disease and patient outcome.

NGAL has previously been reported as a player chiefly in renal and also in several extra-renal diseases, including abdominal aortic aneurysms (18); pre-eclampsia (19); rheumatoid arthritis (20); nasal polyps (21); and neoplasms, including breast (22), gastric (23), colon (24), and pancreatic adenocarcinomas (12). In patients with heart failure, serum NGAL levels were found to be significantly elevated in patients with both acute post-myocardial (MI) and chronic heart failure compared to healthy subjects (25). Significantly, a high baseline NGAL level in patients who developed heart failure immediately after an attack of acute MI was associated with a greater incidence of composite adverse effects, including nonfatal MI, death from cardiovascular causes, overall mortality (from any cause), and stroke. Further, elevated serum NGAL levels were observed even in those heart failure patients in whom the serum creatinine was within normal limits suggesting an extrarenal source for NGAL in this condition. NGAL expression was also elevated among patients with angiographically confirmed coronary artery disease (compared to those with normal coronary arteries) and showed a modest positive correlation with the number of diseased coronary vessels (26). A multivariate analysis revealed that serum NGAL level was an independent predictor of the presence and severity of coronary artery disease in these patients. NGAL also appears to have a function in the pathogenesis of MI itself. In a study, Hemdahl *et al.* (27) using the atherosclerosis prone apolipoprotein E^{-/-} × LDL receptor^{-/-} (apoE^{-/-} × LDLR^{-/-}) mice found that after exposure to brief hypoxic stress, there was a significant increase in the expression of 24p3 (the mouse homolog of NGAL) in the myocardium of the apoE^{-/-} × LDLR^{-/-} mice compared to the wild-type mice. The increase in 24p3 was specific to those mice that developed an acute MI, suggesting that either 24p3/NGAL was released in response to an infarction or that NGAL was itself responsible for the infarction. It has been suggested that NGAL by forming a dimer with the matrix metalloproteinase-9 (MMP-9) protects it from degradation by tissue inhibitor of metalloproteinases. Thus, MMP-9 exerts a proteolytic activity at the site of the atherosclerotic plaque, which in turn promotes disruption of the plaque and leads to an MI.

However, NGAL is perhaps best known for being an early marker of renal injury, both acute and chronic. A prospective study to determine the function of NGAL in early identification of acute kidney injury (AKI) found that among children undergoing cardiopulmonary bypass, urine NGAL levels increased nearly 15-fold within the first 2 h after the procedure among those children who developed AKI (defined as ≥50% increase in serum creatinine) but not in those without this complication (28). By comparison, the rise in serum creatinine was delayed, rising only 2–3 days after cardiopulmonary bypass. At a cutoff of 100 ng/ml, this study also found that a single urine NGAL measurement (by ELISA) was 82% sensitive and 90% specific for predicting AKI among children undergoing cardiopulmonary bypass within 2 h after the procedure. Further, higher 2 h urine NGAL levels correlated with a greater severity and duration of AKI, a longer hospital stay, a requirement for dialysis, and higher mortality. Among patients undergoing liver transplantation, the difference between NGAL levels at baseline and that measured 2 h after reperfusion was observed to be highly predictive of subsequent AKI (29). Further, for patients with serum creatinine <1.5 mg/dl, a single measurement of serum NGAL obtained 2 h after reperfusion of the liver was predictive of a subsequent AKI with an AUC equal to 0.79 (cutoff, 139 ng/ml). AKI in this study was defined as an increase in serum creatinine of ≥50% over baseline within 48h after the surgery. Measurement of NGAL levels (expressed as µg/g of serum creatinine) by immunoblotting in a single urine sample was also found to effectively discriminate AKI from pre-renal azotemia and chronic kidney disease with a high sensitivity and specificity, significantly superior to other markers of AKI, such as serum creatinine, fractional sodium excretion, α1-microglobulin, α1-acid glycoprotein, and *N*-acetyl-

β -D-glucosaminidase (30). Among critically ill patients, urine NGAL levels at the time of admission were observed to be significantly higher in those who developed AKI within the first 48 h (compared to those who did not) (31) and urine NGAL emerged as an independent predictor of the severity of AKI (measured as the time to first dialysis) in these patients. Similar findings were echoed in patients with unstable angina undergoing percutaneous coronary intervention in whom development of contrast nephropathy was associated with an early rise of serum NGAL (within 2 h of the procedure) (32). Interestingly, serum creatinine levels remained largely unaffected during this period, supporting other observations that there is a lag of 2–3 days between the onset of AKI and the rise in serum creatinine. NGAL has emerged as a general marker of renal damage, not just due to acute causes, but even chronic, long-standing pathologies. In one study, NGAL levels were significantly higher in nondiabetic patients with chronic kidney disease (CKD) compared to controls and increased progressively with increasing stage of CKD. Further, on multiple regression analysis, serum creatinine, high-sensitivity CRP, and calcineurin emerged as predictors of serum NGAL levels in nondiabetic patients with CKD. A recent meta-analysis assessing the performance of NGAL as a predictor of AKI found that urine NGAL was as good a predictor as serum NGAL (33). At a cutoff of >150 ng/ml, urine NGAL was a good discriminant marker between those with and without AKI (AUC, 0.815). NGAL was also a better predictor of AKI among patients receiving radio contrast infusion (AUC, 0.89) than in other groups, such as postcardiac surgery (AUC, 0.77) and critically ill patients (AUC, 0.73). NGAL levels also predicted the need for starting renal replacement therapy and in-hospital mortality among patients with AKI.

Animal models have been widely used to understand the time course of inflammatory changes in AP (summarized in a recent article (34). de Dios *et al.* (35) using a rat model of AP observed that activation of immune cells, chiefly neutrophils and monocytes, occurred as early as 1.5 h after surgical ligation of the CBD and was paralleled by an increase in the total leukocyte count (increased in the first 6 h, then declined (at 12 h), finally reaching baseline levels by 48 h). In our animal model, NGAL levels were significantly elevated in mice with both MAP and SAP (compared to control animals) at 6 h. However, 48 h after induction of pancreatitis, NGAL levels returned close to control levels in the mice with MAP, whereas it remained significantly elevated in those with severe necrotizing pancreatitis. Although it is almost certain that circulating neutrophils contribute in part to the increase in NGAL levels observed in the mice and human subjects with AP in our study, we cannot rule out the release of NGAL from other sources including the kidney or from neutrophils that have migrated into the inflamed pancreatic tissue. Injection of lipopolysaccharide (LPS), the major virulence factor in gram-negative bacteria, led to a nearly 15-fold increase in expression of 24p3 (mouse homologue of NGAL) mRNA in the liver (36). This increase, apparent as early as 4 h after administration of LPS, was associated with an increase in 24p3 expression by hepatic macrophages (has a key function in the immune response against LPS). Further, 24p3 mRNA expression also increased in the lungs of these mice within 1 h of administration of LPS. Given our observation that serum NGAL level was elevated even in patients with low serum creatinine, it is possible that the NGAL elevation is due to its release from the liver, lungs, the stressed myocardium, and the macrophages in addition to the kidneys. In support of a function for the distressed kidney in the elevation of serum NGAL levels, we observed a parallelism between serum NGAL and serum creatinine levels when comparing patients with SAP with fatal outcome with those who recovered subsequently. However, NGAL levels were also observed to be significantly higher in patients who developed MOF, a syndrome encompassing dysfunction of the lungs, liver, intestines, and the brain in addition to the kidneys. Unraveling the source(s) for the elevated NGAL levels in patients with SAP with MOF and its relationship to specific organ damage will be of immense clinical benefit in predicting patient prognosis.

A possible explanation for elevation of NGAL levels in AP is an induction of its expression by inflammatory cytokines. A study by Cowland *et al.* (37) showed that treatment of a type-II

pneumocyte-derived cell line with IL-1 β resulted in a nearly 10-fold upregulation of NGAL expression. This effect was specific for IL-1 β as treatment with tumor necrosis factor- α , IL-6, or LPS had no effect on NGAL expression (37). Norman *et al.* had reported earlier that induction of pancreatitis in mice was associated with an early elevation of IL-1 β and tumor necrosis factor- α (38). They also showed that although IL-1 β was subsequently produced in large amounts in the lungs, liver, and spleen in established pancreatitis, the pancreas was the first organ to secrete cytokines after onset of pancreatitis (both mild and severe). They suggested that the pancreas produces cytokines initially in response to the acute inflammation with there being a significant delay in cytokine production by other organs affected by multiorgan dysfunction. In our study, one patient with SAP (designated case 1) who died during the course of illness had nearly ninefold higher serum NGAL level than the mean level of three other SAP cases who recovered. We speculate that a late release of NGAL from the damaged organs was responsible for the sustained levels of NGAL in this patient. The source(s) of NGAL in AP, and specifically the difference in sources for NGAL between MAP and SAP, would offer insight into the function of NGAL in the pathophysiology of AP and the associated organ dysfunction.

It is difficult to identify the time course of biomarkers in patients with AP, chiefly owing to the time lag between the onset of symptoms and the first presentation of the patient at the clinic. Several studies have used animal models, similar to ours to examine the possible function of specific markers in the early detection of severe inflammation of the pancreas and its associated complications. Granell *et al.* (39) observed that in rats with SAP, tumor necrosis factor- α levels (both free and total) increased within 1 h of induction of pancreatitis but then either declined (free) or remained elevated for up to 9 h (total). In our animal model experiment, although NGAL levels increased in mice with both mild and severe necrotizing pancreatitis, a statistically significant increase was noted from 6 to 24 h after induction only in the mice that developed SAP. At 48 h after onset of AP, NGAL levels were still significantly high in the SAP group, whereas it had declined to near baseline levels in the MAP group. Among patients with SAP, NGAL levels in serum reached a peak 48 h after admission, declining progressively thereafter. Although our data on time course of NGAL in patients were restricted by the lack of samples in the first 48 h, there was a good correlation between the pattern of serum NGAL levels in both mouse and human samples, suggesting that NGAL elevation occurs very early during the course of AP, and progressively increasing NGAL levels could indicate impending complications.

Although most studies on biomarkers in SAP have focused on early diagnosis, there are relatively few studies examining correlation of biochemical markers in serum, plasma, or urine with outcome in patients with pancreatic inflammation. A notable study that examined the time course of serum MMP-9, the binding partner for NGAL, in relation to the development of pulmonary complications in rats found that the peak of serum MMP-9 occurred at 6 h in animals who developed SAP (40). This coincided with the first appearance of histological signs of pancreatic damage in these animals. Significantly, pancreatic damage preceded pulmonary damage by nearly 6h, and correlated with declining MMP-9 levels. The animals in this study were followed for up to 72h and a significant elevation of MMP-9 was observed in the SAP group at all time points. Yu *et al.* (41) reported that the expression of human leukocyte antigen (HLA)-DR on peripheral monocytes was inversely correlated with the severity of AP as assessed by the APACHE-II and multiple organ dysfunction scores. There was an inverse relationship between the levels of HLA-DR and that of the inflammatory molecules IL-6 and CRP. Further, a persistent suppression of HLA-DR (defined as $\leq 40\%$ HLA-DR expression) and a second decrease at 14 days after the onset of AP (the lowest level of HLA-DR initially occurred on day 3 of SAP followed by a recovery) correlated with occurrence of septic complications and a poor outcome in patients with SAP (nonsurvivors had $<30\%$ HLA-DR

expression from day 7 onward and never showed a recovery in their HLA-DR levels compared to survivors).

The findings from our pilot study suggest that NGAL could be a potential predictor of MOF in patients with SAP. A significant observation in this study was that the three patients who died during the course of SAP had significantly higher NGAL levels than patients with SAP who survived and had samples collected at the same time point. This observation suggests that NGAL could have a highly significant function as a novel prognostic marker in patients with SAP. Future studies with larger cohorts are needed to evaluate in depth the prognostic significance of elevated serum NGAL levels, specifically in predicting MOF in SAP. Its performance alone and in combination with existing predictors of outcome will also need to be evaluated further.

Although our study unearthed NGAL as a novel diagnostic and prognostic indicator in SAP, this study is based on a relatively small number of samples recruited at a single center. A large, multicenter trial would be the next step to evaluate the scope of these findings particularly the function of NGAL in serum (and urine) as a predictor of MOF in all critically ill patients. Such a marker would be immensely useful to recommend patients for early institution of specialized care. Although we examined NGAL in serum samples grouped according to the time of collection, serial measurements in the same patient over the period of their hospital stay were not undertaken. Future studies will aim toward addressing this shortcoming, together with examination of a possible correlation between NGAL levels and other complications of SAP including pancreatic necrosis.

In conclusion, the early recognition of patients with SAP is an important goal in the optimal management of patients with AP. In this study, we identified in a mouse model of AP that serum NGAL levels rise very early (within 6h) after the onset of AP. Further, we observed in a pilot study that early measurement of NGAL in serum could distinguish SAP from MAP cases with high sensitivity and specificity. Serum NGAL measurement had a high negative predictive value (> 95%) even up to 5 days after the onset of symptoms, suggesting that it could be very useful as a screening test in patients with established AP to exclude the presence of severe disease. Finally, high NGAL levels appeared to predict a poor outcome in patients with SAP. The identification of a novel predictor of severity and outcome in AP could significantly facilitate early prognostication of patients for early initiation of high dependency or intensive treatment in specialized units.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Severe acute pancreatitis is responsible for significant morbidity and mortality.
- ✓ There is no established biomarker for early diagnosis of severe pancreatitis.

WHAT IS NEW HERE

- ✓ Serum NGAL is an early marker of severe pancreatitis being elevated within 48h of onset of symptoms.
- ✓ Elevated NGAL levels are associated with multiorgan failure and fatal outcome in severe pancreatitis patients.

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Figure 1.

Mean ($\pm$ s.e.) levels of NGAL/lipocalin-2 in sera of mice after the induction of acute pancreatitis. Sixty mice were randomized into three groups of 20 animals each. The mice were infused for 5 min with 4% sodium taurocholate (induces severe pancreatitis) or isotonic saline (induces mild edematous pancreatitis) into the CBD. Five mice from each treatment group were killed 6, 12, 24 and 48h after treatment and the serum samples were collected. Control mice injected with isotonic intraperitoneal saline do not develop any features of acute pancreatitis. The level of NGAL in the serum was quantified by enzyme-linked immunosorbent assay. **(a)** Mean serum NGAL levels ($\pm$ s.e.) in mice with severe, mild, or no pancreatitis. NGAL levels were significantly higher in mice with severe pancreatitis compared to those with mild pancreatitis at 24 ($P=0.003$) and 48h ($P=0.03$) but not at 6 or 12h after induction. **(b)** Representative histology (H&E-stained sections) of the pancreas from control mice (left), mild pancreatitis (middle), and severe pancreatitis (right). Severe pancreatitis is characterized by marked inflammatory infiltration (arrows) and acinar cell necrosis (*), while these changes are rarely observed (necrosis) or significantly attenuated (inflammation). The duodenum is labeled as $\odot$. Original magnification 1:25. CBD, common bile duct; H&E, hematoxylin and eosin; NGAL, neutrophil gelatinase-associated lipocalin.

Figure 2.

Comparison of serum NGAL, CRP, Ranson's score, peak APACHE-II score, and serum creatinine in patients with SAP with a fatal vs. nonfatal outcome. To examine the ability of serum NGAL levels to predict outcome, we compared NGAL levels in patients with SAP who died during the course of their illness with patients with SAP who survived. Cases were grouped according to the time the earliest serum sample was available after the onset of symptoms. Three patients with SAP who died during hospitalization were designated as case 1 (a), case 2 (b), and case 3 (c) (labeled as "dead" in the figure). For comparison, patients with SAP who had serum samples drawn at the same time point but who survived (labeled as "alive") were chosen. Prognostic indicators, including plasma CRP, the Ranson's score at 48h, the peak

APACHE-II score, serum creatinine and serum NGAL levels, were compared between the two groups of patients (alive vs. dead). NGAL levels were markedly higher in the “dead” patients with SAP (9.2-, 5.3-, and 1.5-fold higher in cases 1, 2, and 3, respectively) compared to the mean NGAL level in the “alive” group. Patients with SAP who developed MOF are indicated by the asterisk (*) beside the patient number. NGAL concentrations in the graphs are 1/100th of the actual concentration for the respective patients. APACHE-II, Acute Physiology and Chronic Health Evaluation-II; CRP, C-reactive protein; MOF, multiorgan failure; NGAL, neutrophil gelatinase-associated lipocalin; SAP, severe acute pancreatitis.

Table 1

Serum NGAL levels in mice with experimentally induced AP

Time (h)	Severe necrotizing pancreatitis (ng/ml)	Mild edematous pancreatitis (ng/ml)	Control mice (ng/ml)	P value (mild vs. severe pancreatitis)
6	436±52 (n=5)	724±22 (n=5)	73±17 (n=5)	0.44
12	1,199±42 (n=5)	883±47 (n=5)	143±17 (n=5)	0.7
24	1,670±20 (n=5)	700±61 (n=5)	73±17 (n=5)	0.003
48	1,497±37 (n=5)	158±17 (n=5)	25±17 (n=5)	0.03

AP, acute pancreatitis; NGAL, neutrophil gelatinase-associated lipocalin.

Table 2

Characteristics of patients with AP included in the study

	MAP=28	SAP=16	P value
Age, mean ($\pm$ s.e.)	55.5 ($\pm$ 3.35)	51.3 ($\pm$ 4.57)	0.68
Gender (male/female)	12/16	12/4	0.03
Race (% of Caucasians)	82%	87%	0.642
Length of stay (days)	8 ($\pm$ 1.1)	20.8 ($\pm$ 2.4)	<0.0001
BMI	30.1 ($\pm$ 1.5)	30.4 ($\pm$ 1.2)	0.87
<i>Etiology (% of total)</i>			
Gall stones	8 (29)	6 (37)	
Alcohol	1 (4)	3 (19)	
Idiopathic	11 (39)	2 (12)	
Post-ERCP	5 (18)	2 (12)	
Hypertriglyceridemia	2 (7)	2 (12)	
Pancreatic necrosis	3 (10.7)	9 (56.2)	0.0011
Ranson's score at 48h	1.5 $\pm$ 0.25	5.3 $\pm$ 0.5	0.0001
Peak APACHE-II	7.4 $\pm$ 0.97	14.4 $\pm$ 1.5	0.0001
APACHE-II score at admission	5.9 $\pm$ 0.9	10.6 $\pm$ 1.3	0.007
C-reactive peptide (pg/ml)	7.4 $\pm$ 0.4	9.4 $\pm$ 0.4	0.0007

AP, acute pancreatitis; APACHE-II, Acute Physiology and Chronic Health Evaluation-II; BMI, body mass index; ERCP, endoscopic retrograde cholangiopancreatography; MAP, mild acute pancreatitis; SAP, severe acute pancreatitis.

Table 3Mean ($\pm$ s.e.) NGAL levels in SAP and MAP according to the time of collection of the sample

Time point (h)	SAP (ng/ml)	MAP (ng/ml)	<i>P</i> value
48	1,314 $\pm$ 151	90 $\pm$ 10	0.003
72	332 $\pm$ 209	83 $\pm$ 9	0.32
96	359 $\pm$ 173	72 $\pm$ 14	0.16
120	211 $\pm$ 77	125 $\pm$ 9	0.31

MAP, mild acute pancreatitis; NGAL, neutrophil gelatinase-associated lipocalin; SAP, severe acute pancreatitis.

Table 4

Sensitivity, specificity, PPV, and NPV of serum NGAL in distinguishing SAP from MAP

Time of obtaining first sample (n)	Cutoff (ng/ml)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	AUC (95% CI) P value
≤5 Days (SAP, 12; MAP, 27)	> 113.6	94% (69.7–99)	84% (68.7–94)	71% (47.8–88.6)	94.1% (80.3–99.1)	0.92 (0.81–0.98), P=0.0001
≤4 Days (SAP, 11; MAP, 25)	> 125.6	92% (61.5–98.6)	97% (84.6–99.5)	92% (61.5–98.6)	97% (84.6–99.5)	0.93 (0.81–0.98), P=0.0001
≤3 Days (SAP, 7; MAP, 19)	> 125.6	87.5% (47.4–98)	96% (81.6–99.4)	87.5% (47.4–98)	96.4% (81.6–99.4)	0.89 (0.74–0.97), P=0.0001
≤2 Days (SAP, 4; MAP, 10)	> 175.3	100% (40.2–100)	100% (82.2–100)	100% (40.2–100)	100% (82.2–100)	1.0 (0.813–1.00), P<0.0001

AUC, area under the curve; MAP, mild acute pancreatitis; NGAL, neutrophil gelatinase-associated lipocalin; NPV, negative predictive value; PPV, positive predictive value; SAP, severe acute pancreatitis.

Table 5

Analysis of multiple variables in SAP patients with or without MOF

Factor	MOF					
	Absent			Present		
	N	Mean (±s.e.)		N	Mean (±s.e.)	P value
Age (years)	5	42±4		6	59±9	0.135
BMI	5	31±1		6	30±2	0.569
Length of stay (days)	5	20±5		6	17±5	0.435
Serum creatinine at admission (mg/dl)	5	1±0.1		6	2±0.5	0.843
Peak serum creatinine (mg/dl)	5	1±0.1		6	3±1	0.024
Serum amylase (U/l) ^a	4	862±527		6	722±232	0.648
Serum lipase (U/l) ^a	4	3,718±2,303		6	4,087±1,375	0.986
Serum NGAL (ng/ml)	5	179±42		6	906±195	0.004
Serum CRP (pg/ml)	5	9±1		6	9±1	0.947
Admission APACHE-II score	5	7±2		6	13±2	0.133
Peak APACHE-II score	5	10±2		6	19±2	0.008
Ranson's score (at 48h)	5	4±1		6	7±1	0.121

APACHE-II, Acute Physiology and Chronic Health Evaluation-II; BMI, body mass index; CRP, C-reactive protein; MOF, multiorgan failure; NGAL, neutrophil gelatinase-associated lipocalin; SAP, severe acute pancreatitis.

^a Amylase and lipase levels were available for 4 out of 5 SAP patients who did not have MOF.